Vertebrate MAX-1 is required for vascular patterning in zebrafish

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During embryogenesis, stereotypic vascular patterning requires guidance cues from neighboring tissues. However, key molecules involved in this process still remain largely elusive. Here, we report molecular cloning, expression, and functional studies of zebrafish *max-1*, a homolog of *Caenorhabditis elegans max-1* that has been implicated in motor neuron axon guidance. During early embryonic development, zebrafish *max-1* is specifically expressed in subsets of neuronal tissues, epithelial cells, and developing somites through which vascular endothelial cells migrate from large ventral axial vessels to form stereotypic intersegmental blood vessels (ISV). Blocking zebrafish *max-1* mRNA splicing by morpholino injection led to aberrant ISV patterning, which could be rescued by injection of either *C. elegans* or zebrafish *max-1* mRNA. Analysis of motor neurons in the same region showed normal neuronal axon pathfinding. Further studies suggested that the ISV defect caused by *max-1* knockdown could be partially rescued by overexpression of *ephrinb3* and that *max-1* was involved in mediating membrane localization of *ephrin* proteins, which have been shown to provide guidance cues for endothelial cell migration. Our findings therefore suggest that *max-1*, acting upstream of the *ephrin* pathway, is critically required in vascular patterning in vertebrate species.

ephrin

In vertebrates, the first blood vessels that develop are the large midline artery and vein, formed through vasculogenesis involving migration, differentiation and assembly of angioblasts (1). After the establishment of the major axial artery and vein structures, smaller blood vessels such as the trunk intersegmental vessels (ISV) are formed through angiogenesis, which involves the sprouting of endothelial cells from the preformed large vessels. The patterning of smaller angiogenic vessels is highly stereotypical and appears conserved among vertebrate species. Our understanding of angiogenic patterning during development has largely come from studies of model organisms, including chick, mouse, *Xenopus*, and zebrafish. In zebrafish, the first angioblasts arise in the posterior lateral mesoderm (2) and then migrate medially to form the primordia of the large axial vessels, the dorsal aorta and vein. Subsequently, endothelial cells migrate from the dorsal aorta to generate the secondary angiogenic ISV in the trunk region (3).

Both vascular and angiogenic development appear to involve signals from adjacent tissues. For instance, the notochord plays a key role in blood vessel development (4–6). In the zebrafish mutants *floating head* and *sonic-you*, notochord defects result in the absence of a dorsal aorta and ISV fail to sprout. Recently, a number of axon guidance molecules have been shown to be involved in vascular patterning. These molecules include the semaphorins, *sema3a1* and *sema3a2* (7), netrin (8, 9), robo (10), neuropilin (11), and ephrin (12). These studies demonstrate a remarkable similarity between the molecular mechanisms that regulate neuronal and endothelial cell migration and patterning (13).

Max-1 was first identified and characterized as a neuronal guidance gene in *Caenorhabditis elegans*; its loss of function caused variable axon pathfinding defects (14). The *C. elegans* MAX-1 protein contains PH, MYTH4, and FERM domains

arranged from N terminus to C terminus. This combination of domains suggests that MAX-1 represents a previously uncharacterized class of proteins. Huang *et al.* (14) also identified predicted homologs of *max-1* gene in other species, including *Drosophila*, mouse, and human. It was further shown that human *max-1* could partially rescue the *C. elegans max-1* mutant phenotype, and RNA whole-mount *in situ* hybridization using a partial cDNA probe from mouse *max-1* revealed primary expression of *max-1* in the nervous system and muscle tissue of mouse embryos. These observations suggest that *max-1* is conserved from *C. elegans* to human and that it might also function in the neural development of vertebrates.

We isolated the homolog of *max-1* from zebrafish and analyzed its embryonic expression and function. Similar to its expression in mouse, zebrafish *max-1* is mainly expressed in neuronal tissues and somites during development. An anti-sense morpholino oligo (MO) targeting a splice-site of zebrafish *max-1* mRNA was designed, and embryos injected with this morpholino (morphant) were shown to lack a functional transcript of *max-1*. First, neuronal pathfinding in the morphant was examined by using transgenic zebrafish that have the somitic motor neurons labeled with specific GFP expression, but no apparent defects were observed. Because a number of neuronal guidance molecules have been implicated in vascular endothelial patterning, we examined ISV in the morphants of another transgenic zebrafish line that expresses GFP in blood vessels. Interestingly, abnormal ISV patterning was indeed observed, and the defect was specific to *max-1* knockdown, because it could be rescued by mRNA injection of either *C. elegans* or zebrafish *max-1*. The ISV defect could also be partially rescued by overexpression of *ephrinb3*. In addition, *max-1* appears to regulate membrane localization of *ephrin* proteins; the *ephrinb3*-GFP fusion protein becomes abnormally aggregated in cytoplasm of *max-1* knockdown cells. Our findings therefore suggest that *max-1*, acting epistatically upstream of *ephrin* pathway, is critically required in vascular patterning in vertebrate species.

Results

Molecular Cloning and Sequence Analysis of Zebrafish *max-1*. A Blast search (15) of the Ensembl zebrafish genomic sequence database showed that zebrafish has one copy of the *C. elegans max-1* homolog that is comprised of at least 28 exons on chromosome 17. Two nearby gene markers, XP.689775.1 and Q5BL95.BRARE, were colocalized with *max-1* on one chromosome in human, mouse, and

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Abbreviations: DLAV, dorsal longitudinal anastomotic vessels; dpf, days postfertilization; hpf, hours postfertilization; ISV, intersegmental blood vessels; MO, morpholino oligo.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. DQ501275).

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Fig. 3. Location of splice-blocking MO1 and MO2 and the resulting zebrafish *max-1* splicing variants. Above the gel is the putative protein structure of zebrafish *max-1*. The PH domain corresponds to exons 10, 11, and 12. The gene structure of zebrafish *max-1* from exon 5 to exon 14 is shown. MO1 binds to the exon 9/intron 9 boundary, and MO2 targets the exon 8/intron 8 boundary. In morphant, no wild-type mRNA (band a) was detected by RT-PCR. Band a is wild-type mRNA. Bands b and c lose part of coding sequence (black shadows) and result in a premature stop codon before the PH domain.

staining in the brain began to disappear (Fig. 2H), and, at 28 hpf, expression in the brain was remarkably reduced (Fig. 2I and J). *Max-1* expression in the ear persists up to 5 days postfertilization (dpf) (Fig. 2K).

In epithelial tissues. At the 14-somites stage, the distal edge of the tail bud began to show weak expression of zebrafish *max-1* (Fig. 2F); this staining was stronger at the 18-somites stage. As embryogenesis continued, this staining extended anteriorly along the dorsal edge of embryo. At the 21-somites stage, staining of the dorsal midline was observed (Fig. 2G); in transverse sections, this staining corresponds to epithelial tissue (data not shown). Expression in the dorsal midline disappears at 33 hpf. Other epithelial tissues that expressed zebrafish *max-1* were the lining of the mouth (from the 26-somites stage to 3 dpf) (Fig. 2H), the edge of pectoral fin bud (36 hpf to 3 dpf) (Fig. 2J), and the edge of the operculum at 3 dpf (data not shown).

Knockdown of Zebrafish *max-1* Resulted in an ISV Patterning Defect.

To study function of *Max-1*, two MOs (MO1 and MO2) were designed to block *max-1* splicing (Fig. 3). MO1 is complementary to the putative exon 9/intron 9 boundary, and MO2 is complementary to exon 8/intron 8 boundary. Respectively, 5.2 ng of MO1 and MO2 or equal amount of each in combination was injected into the 1- to 2-cell-stage, *flk-1:GFP*-transgenic zebrafish embryos (morphant), and development of GFP-labeled blood vessels in the live transgenic zebrafish embryos was directly observed under a fluorescent microscope for 5 days. At this dosage, although morphants appeared slightly thinner than the uninjected controls little other general morphological defects were observed. At higher dose, 10.4 ng each of MO1 and MO2 per embryo, morphants showed general developmental defects, including a small head and curled trunk (data not shown).

Specificity and efficiency of splice-blocking were determined by RT-PCR with primers corresponding to exons flanking the MO target sites. Although injection of each MO reduced the

max-1 transcript (data not shown), coinjection of MO1/MO2 resulted in complete loss of the expected wild-type fragment (1.8 kb; Fig. 3, band a) and generation of two additional smaller bands (Fig. 3, bands b and c). Sequencing showed that abnormal transcripts had lost exon 9 for the band b and exon 8 and exon 9 for band c (Fig. 3). The resulting aberrant mRNA transcripts contained translational frame shifts and premature stop codons ahead of the PH domain, suggesting that no dominant-negative proteins were formed. Zebrafish *ef-1 α* was used as an RT-PCR control, showing that equal amount of PCR product was amplified, and no abnormal splicing was observed (Fig. 3).

At 1 dpf, injection of MO1 and MO2 resulted in missing or misdirected ISVs (Fig. 4B). Injected embryos develop slightly slower than the control group, but overall embryonic structures appeared normal. As development continued, those ISV missed at 1 dpf eventually sprouted from the dorsal aorta, but most did not follow the normal stereotypical intersomitic pathway, following instead an irregular network (Fig. 4D). At 3 dpf, $\approx 83.7\%$ of the morphants still showed an aberrant ISV pattern that persisted until at least 5 dpf, the last time point observed (Fig. 4L).

To confirm the specificity of MO1 and MO2, zebrafish or *C. elegans* *max-1* mRNA was coinjected with the morpholino to determine whether they could rescue the morphant phenotype. As shown in Fig. 4, both zebrafish and *C. elegans* *max-1* mRNA rescued the morphant ISV phenotype (Fig. 4E and F). Of the embryos injected with zebrafish *max-1* mRNA, 60.8% showed complete rescue and 26.9% showed partial rescue. Of the embryos injected with *C. elegans* *max-1*, 33.6% showed complete rescue and 50.1% showed partial rescue (Fig. 4L).

We then performed microangiography with TRITC to determine whether the aberrant ISV could support circulation. As shown in Fig. 4G and H, ISVs that connected to both the dorsal aorta and the dorsal longitudinal anastomotic vessels (DLAV) contributed to blood circulation, whereas truncated ISV blocked circulation.

The effect of MO1 and MO2 was specific to vascular structures because the somites and motor neuron patterning in the trunk region appeared normal. The motor neurons were visualized in live *gata2:GFP* transgenic zebrafish embryos (Fig. 4I and J). In zebrafish injected with the higher amount of morpholino (10.2 ng each of MO1 and MO2), motor neuron axons also started to show abnormal patterns (data not shown).

***Max-1* Genetically Interacts with *ephrinb3* in Zebrafish.** Several neural guidance molecules have been shown to be involved in patterning of angiogenesis. Previous studies suggested that *sema3Ab* (16), *ephrinb1*, *ephrinb2a*, *ephrinb2b*, and *ephrinb3* (17) were expressed in the zebrafish somites during ISV formation. We therefore selected these genes as candidates for potential rescue of the *max-1* morphant phenotype by coinjection of their mRNA transcripts with the zebrafish *max-1* MOs. *Ephrinb3* was the only gene tested that could partially rescue a *max-1* morphant (Fig. 4K and L).

From 14 hpf to 24 hpf, *ephrinb3* is expressed in the ventral somites and ventral mesenchymal cells, a similar expression pattern to *max-1* in somites. Biochemical studies have established that *ephrin* family genes encode transmembrane proteins. To test whether *max-1* plays a role in mediating the subcellular localization of the *ephrinb3* protein, we produced an *ephrinb3*-GFP fusion protein construct and confirmed that this fusion protein indeed was predominantly localized on the plasma membrane of cells from dissociated embryos. Coinjection of mRNA encoding the *ephrinb3*-GFP fusion protein and MO1 plus MO2 resulted in abnormal distribution of the *ephrinb3*-GFP fusion protein in cells isolated from injected embryos at 24 hpf. Compared with control-injected embryos, the fusion protein formed more aggregates in cytoplasm of cells from morphant embryos (Fig. 5). This effect was specific to *ephrinb3*-GFP fusion

Fig. 4. Zebrafish *max-1* knockdown causes defects in ISV patterning but not in neuronal axon guidance. Embryos are anterior to the left and dorsal to the top. *A–H* and *K* are *flk-1:GFP* embryos. *I* and *J* are *gata2:GFP* embryos. (*A*) Control-injected embryo at 1 dpf. (*B*) *max-1* knockdown morphant at 1 dpf. The white star indicates a missing ISV, and the white arrowhead points to an aberrant ISV. (*C*) Control embryo at 3 dpf. (*D*) *max-1* knockdown morphant at 3 dpf. Most ISVs are aberrantly patterned. (*E*) Zebrafish *max-1* rescue. The MOs were coinjected, with 24 pg of zebrafish *max-1* mRNA per embryo. ISV patterning appears normal. (*F*) *C. elegans max-1* rescue. The MOs were coinjected, with 24 pg of *C. elegans max-1* mRNA per embryo. (*G*) *max-1* morphant at 2 dpf. White arrowheads point to the ISVs that do not connect to the DLAV. (*H*) Angiography with TRITC in the same embryo in *G*. White arrowheads point to the same ISVs as in *G*. No circulation was observed at those two positions. (An embryo with a less severe phenotype was used to compare connected and nonconnected ISVs.) (*I*) *gata2:GFP* control embryo at 30 hpf. The motor neuron axon pattern is normal. (*J*) *max-1* MO knockdown in a *gata2:GFP* embryo at 30 hpf. The motor neuron axon pattern is normal. (*K*) Partial rescue of morphant ISV phenotype with zebrafish *ephrinb3* mRNA:MO coinjected, with 32 pg of zebrafish *ephrinb3* mRNA per embryo. Most ISVs connect to the DLAV. However, some still show an aberrant pattern. (*L*) Quantitative comparison of zebrafish *max-1* knockdown by MO1 and MO2 coinjection and phenotypic rescue at 3 dpf. Of the morphant embryos

Fig. 5. ephrinb3-GFP fusion protein localization in control and *max-1* morphant zebrafish cells. Control cells contained 47 pg of ephrinb3-egfp mRNA, memb-mCherry mRNA, and 10.2 ng of MAX1MO4mis per embryo. Both GFP and red fluorescent protein (RFP) signals are evenly localized on the cell membrane. MAX1MO4mis is a control MO that has no phenotype at this concentration. Morphant cells contained 47 pg of ephrinb3-egfp mRNA, memb-mCherry mRNA, and 5.2 ng of MO1/MO2 per embryo. GFP expression aggregates are shown in the cytoplasm, whereas RFP is evenly localized on membrane. (*A* and *B*) Detection under GFP filter. (*C* and *D*) Detection under RFP filter. (*E* and *F*) Superimposition of GFP and RFP images. White arrowheads in *B* and *F* point at GFP aggregation. (*G*) Quantitative comparison of control and morphant. Of the control cells ($n = 550$), 68.7% have a GFP signal only on the membrane and 31.3% have a GFP signal both on the membrane and in the cytoplasm. Of the morphant cells ($n = 551$), 49.5% have a GFP signal only on the membrane, and 50.5% have a GFP signal both on the membrane and in the cytoplasm. In the key for *G*, membrane means that the GFP signal is only on membrane, and cytoplasm means that the GFP signal is both on the membrane and in the cytoplasm. Error bars show standard errors ($P < 0.001$).

protein because subcellular localization of RFP membrane protein, an internal control, remained normal in the same cells where ephrinB3-GFP was affected.

Discussion

In *C. elegans*, *max-1* is involved in motor neuron axon guidance and acts genetically downstream of the netrin pathway (14). In this report, we identified and analyzed expression and function of the *max-1* gene in zebrafish. Embryos that had been injected with two

($n = 106$), 83.7% showed a defective phenotype, and 16.3% showed a normal phenotype. Of the zebrafish *max-1* (*z.max-1*) rescue embryos ($n = 109$), 12.3% showed a defective phenotype, 60.8% were rescued (normal), and 26.9% were partially rescued. Of the *max-1* rescue embryos ($n = 122$), 16.3% showed a defective phenotype, 33.6% were rescued (normal), and 50.1% were partially rescued. Of the *ephrinb3* rescue embryos ($n = 135$), 23.1% showed a defective phenotype, 18.4% were rescued (normal), and 58.5% were partially rescued. Error bars show the standard errors ($P < 0.001$).

splice-blocking MOs (MO1 and MO2; 5.2 ng each per embryo) lacked functional *max-1* mRNA transcripts yet developed without any apparent general defects. Approximately 83.7% of these morphants exhibited abnormal ISV patterning (Fig. 4*L*). Fish injected under the same conditions showed no obvious defects in motor neuron axon guidance (Fig. 4*I* and *J*). In zebrafish injected with the higher amount of morpholino (10.2 ng each of MO1 and MO2), both ISVs and motor neuron axons appeared abnormally patterned. It is therefore possible that MAX-1 also plays a role in neuronal axon guidance in vertebrates.

We focused on analysis of the more specific function of *max-1* on ISV patterning seen in zebrafish injected with the lesser amount of morpholino. In control embryos, individual ISVs are restricted ventrally to the border between adjacent somites. At the dorsal–ventral midpoint, the ISV leaves the somite border and runs directly dorsal to connect with the DLAV (3). However, in morphants, ISVs are often truncated or branch out aberrantly, without following a specific pattern, into adjacent somites. We therefore speculated that certain guidance signals might be disturbed in *max-1* morphants.

Several signaling molecules that function in both axon guidance and angiogenesis have been reported in zebrafish, including netrin, semaphorin, robo, neuropilin, and ephrin proteins. To examine whether any of these proteins interact with *max-1*, we selected a few that were reported to be expressed in somites during the same developmental time frame that *max-1* is expressed as candidates that might rescue the *max-1* morphant phenotype. Among these candidates, zebrafish *ephrinb3* was found capable of partially rescuing the ISV phenotype of *max-1* morphants. We confirmed that the expression of *ephrinb3* and *max-1* indeed overlaps in the ventral somites of zebrafish embryos from the 10-somites stage to 26 hpf (Fig. 2 *C*, *F*, *H*, and *L–P*). These studies suggest that *ephrinb3* functions downstream of *max-1* to regulate ISV patterning. Given that ephrin proteins have been implicated in tissue patterning and boundary formation through repulsive guidance cues (18, 19), we propose a hypothesis that ISV patterning in the trunk of zebrafish involves repulsion mechanisms mediated by MAX-1 and the ephrin protein pathway. We have attempted to knock down *ephrinb3* by morpholino injection, but so far the data are inconclusive because of our inability to confirm the effectiveness of the *ephrinb3* morpholino and perhaps because of the functional redundancy of additional ephrin proteins expressed in the somites.

Our hypothesis that ephrin signaling is involved in ISV patterning in zebrafish is consistent with previous studies showing that Eph receptors and ephrins can trigger an adhesive response of endothelial cells and are required for the remodeling of blood vessels (20). Biochemical studies suggest that the extent of multimerization of Eph receptors and ephrins on the cell membrane modulates the cellular response. MAX-1 was shown to be localized beneath cytoplasm membrane mediated by its FERM domain (14). In addition, PH domains are often involved in targeting proteins to the plasma membrane (21). Our studies showed that an *ephrinb3*-GFP fusion protein is normally localized on the membrane and that coinjection of mRNA encoding this fusion protein and MO1 and MO2 caused excess aggregation of GFP signal in cytoplasm. This finding suggests that appropriate membrane association of ephrin proteins may require proper function of MAX-1 in zebrafish. Overall, our studies have identified MAX-1 as a critical component of the guidance pathway underlying endothelial cell migration and blood vessel patterning and further strengthen the similarity between vascular and neuronal developmental regulatory mechanisms.

Materials and Methods

Zebrafish Stocks. The wild-type AB strain zebrafish, *gata2:GFP* (22, 23) and *flk-1:GFP* transgenic (24), was used in this study. Zebrafish embryos were staged as described by Kimmel *et al.* (25).

Molecular Cloning of Zebrafish *max-1* and Phylogeny Analysis. The Ensembl zebrafish database was searched with the translated protein sequence from *C. elegans max-1*, and a predicted protein (ctg10711.1.70473.135987) sharing sequence homology with *max-1* was identified. Primers were designed according to the sequence of ctg10711.1.70473.135987 and used in RT-PCR to isolate the zebrafish *max-1* cDNA from total RNA isolated from 18-somites-stage embryos. In addition, 5'RACE (First Choice RLM-RACE kit, Ambion, Austin, TX) was used to determine the 5' end of the zebrafish *max-1* mRNA, and a full-length putative cDNA clone was constructed. The nucleotide sequence of zebrafish *max-1* was submitted to GenBank, (accession no. DQ501275). Clustalx (version 1.83) was used for the phylogenetic analysis (26).

Whole-Mount *In Situ* Hybridization. Digoxigenin-labeled antisense RNA probes were generated *in vitro* by using the zebrafish *max-1* and *ephrinb3* cDNA as templates with T3 or T7 RNA polymerase (Promega, Madison, WI). Whole-mount RNA *in situ* hybridizations were performed essentially as described by Westerfield (27) on embryos at the following developmental stages: bud, 3 somites, 10 somites, 14 somites, 15 somites, 21 somites, 26 somites, 1 dpf, 28 hpf, 2 dpf, 3 dpf, 4 dpf, and 5 dpf.

Morpholino Design, Phenotype Analysis, and Detection of Splice-Blocking Variants of Zebrafish *max-1* mRNA with RT-PCR. Two splice-blocking MOs of zebrafish *max-1*, MO1 and MO2, were synthesized by Gene Tools (Philomathe, OR). The sequence of MO1 is 5' TAATTCTGTCATCTTACCCAAGAGG3', which corresponds to the putative exon 9/intron 9 boundary of zebrafish *max-1*; the sequence of MO2 is 5'GGAGATGCTGATGTACCTGCAGCAGAGC3', which targets the putative exon 8/intron 8 junction of zebrafish *max-1*.

MO1 and MO2 were dissolved in nuclease-free water at 0.2 mM and injected into 1- to 2-cell-stage embryos (5.2 ng for each oligo per embryo or 2.6 ng of both oligos). The amount of injection was determined by measuring the volume of liquid injected (20 times) into a 1- μ l capillary glass (34 mm long) using a ruler, and volume per microinjection was thereby calculated. Injected embryos were cultured in fish water at 28.5°C.

The defective phenotype was defined as one having five or more ISVs absent or misdirected in the region above the yolk extension and tail. The normal or rescued phenotype was defined as having no more than two ISVs absent or misdirected in the same region. The partially rescued phenotype was defined as having three to four ISVs absent or misdirected in the region from yolk extension to tail.

At 26 hpf, total RNA was extracted from the morphant (MO1 and MO2 coinjected embryos) and control embryos with a Qiagen RNeasy kit. Reverse transcription was carried out with random decamers by using SSII reverse transcriptase (Invitrogen, Carlsbad, CA). Two rounds of PCR were performed to detect splice-blocking variants of zebrafish *max-1* mRNA. The primers used were S6 (base pairs 600–622), 5'-ACATGCAGCCTAGCGAAACAGAA-3'; R11 (base pairs 2,403–2,382), 5'-ATCACGTCACCTCGGCGACTTAT-3'; and R12 (base pairs 2,452–2,431), 5'-GGCAATATGACACGAGGAGTTG-3'. RT-PCR products were cloned and sequenced. Zebrafish *ef-1 α* (GenBank accession no. NM.131263) was used as an internal control. The *ef-1 α* primers used were *ef-1 α* forward (base pairs 496–516), 5'-TCACCCTGGGAGTGAAACAGC-3', and *ef-1 α* reverse, (base pairs 1,188–1,168), 5'-ACTTGCAGGCGATGTGAGCAG-3'. The product size of *ef-1 α* is 692 bp.

A translation-blocking MO, MAX1MO (5'-TCTCTGTTC-CAGCTCCTGCATCTAC-3'), and a 4-bp mismatch control MO, MAX1MO4mis (5'-TCTCTcTTCCAcCTCCtCATg-TAC-3'), were generated by Gene Tools. These oligos were used

as controls for injection because neither of them had any defective phenotype at 10 ng per embryo.

mRNA Preparation. pXT7, a derivative of pGEM4Z (Promega, Madison, WI) and pSP64T (28), was used to make mRNA. A region of the zebrafish *max-1* cDNA containing the coding sequence (base pairs 503-4660) was subcloned into pXT7. The *C. elegans max-1* cDNA clone was provided by Y. Jin (University of California, Santa Cruz); memb-mCherry construct encodes a membrane red fluorescent protein and was a gift from S. Megason (California Institute of Technology, Pasadena); zebrafish *ephrin* cDNA clones (*ephrinb1*, *ephrinb2a*, *ephrinb2b*, and *ephrinb3*) were kindly provided by J. Chan (Children's Hospital, Boston, MA). *Ephrinb3* was subcloned in frame to the 5' end of EGFP (Clontech, Mountain View, CA) to generate a fusion protein. A zebrafish *Sema3Ab* clone was a gift from C. Becker (Zentrum für Molekulare Neurobiologie, Hamburg, Germany). Sense transcripts from the cDNA clones were generated by using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) and were purified with the RNeasy Mini kit (Qiagen, Hilden, Germany).

Microangiography. TRITC dextran at 20 mg/ml (molecular weight 70,000; Research Organics, Cleveland, OH) dissolved in double distilled water was microinjected into the sinus venosus/cardinal vein of zebrafish embryos at ≈ 48 hpf essentially as described by Weinstein and coworkers (29) and Chen *et al.* (30).

Cell Dissociation. Dechorionated embryos were dechorded with a forceps and digested in 0.25% trypsin (Sigma, St. Louis, MO) and 1 mM EDTA in PBS (13.7 mM NaCl/0.27 mM KCl/0.43 mM Na_2HPO_4 /0.14 mM KH_2PO_4 , pH 7.3) at 37°C for 20 min with periodic agitations. Then the cells were centrifuged at $1,000 \times g$ at 4°C for 2 min and washed with PBS two times.

Imaging. Images of zebrafish embryos were acquired by using a Axioplan 2 microscope (Zeiss, Oberkochen, Germany) equipped with a C4742–95 digital camera (Hamamatsu, Tokyo, Japan) or an AxioCam digital camera and OpenLab (Improvision, Lexington, MA) software, then edited with PhotoShop 7.0 (Adobe Systems, San Jose, CA).

Statistical Analysis. Phenotypes of morphants, rescue results by mRNA injections, and cellular sublocalization of the ephrinb3 protein were analyzed by a two-way χ^2 test. The same experiments with similar numbers of embryos or cells were repeated three times.

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